Antioxidants in Clove

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ABSTRACT

Through the use of thin-layer chromatography (TLC), ultraviolet (UV), infrared (IR), mass spectrometry (MS) and high performance liquid chromatography (HPLC), gallic acid and eugenol were identified as the 2 major antioxidants in clove. The amounts of gallic acid and eugenol were determined to be 1.26 g and 3.03 g respectively in 100 g of clove.

INTRODUCTION

The Department of Defense is concerned with increasing the shelf-life of military rations because of multi-year storage needs. This is achieved in some foods by using antioxidants such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT) and Tertiary-Butyl Hydroquinone (TBHQ). Today there is an increasing opposition to the use of synthetic compounds as chemical additives in foods. The search is on for acceptable natural products that can act as antioxidants either alone or synergistically with the chemical additives, thus decreasing the amount of the latter needed.

Despite the knowledge that spices help preserve food, little work has been done on identifying their antioxidants. Chipault (1,2,3,4) in the 1950's investigated the antioxidative properties of spices. In the 1960's Hermann (5,6) isolated an antioxidant in rosemary and sage that he called labiatic acid, and Brieskorn and Doemling (7,8,9) identified carnosol and carnosic acid in the same 2 herbs.

Research increased in the 1970's, especially in Japan, where Hirahara, Takai and Iwao (10) found a variation in the effects of spices depending on the source and type of the spice, time of harvest and treatment, and with slight differences in experimental conditions including the type of food, water content, emulsified states, preservation conditions and method of determining deterioration rates. They went on to investigate the effect of certain spices and spice extracts on soybean, olive, sesame and linseed oils. Watanabe and Ayano (11) prepared and tested water and ethanol soluble fractions as well as the ground state of 10 spices. Yutaka Saito (12) has worked and written extensively on spices, including a review on the progress of research on the antioxidant properties of spices through 1977. He concluded that rosemary, sage, thyme, marjoram and oregano among the herbs and clove, ginger, nutmeg and mace among the spices have strong antioxidation effects. Identification of the substances contained in these spices that cause the effects are limited to those found in rosemary and sage most recently by Chang, Ostric-Matijasevic, Hsieh and Huang (13,14) and Wu, Lee, Ho and Chang (15). Bishov, Henick, et al. (16-21) of these laboratories investigated the oxidation of fat in model systems, the antioxidant effect of the components of freeze-dried foods and the synergism of various antioxidants when used together. Bishov, et al. (22) found antioxidant activity in clove and thyme and so they were picked for this study, which was initiated to identify the compounds responsible for their antioxidant activity.

INSTRUMENTATION AND MATERIALS

The ultraviolet analysis was performed on a Cary Model 15 Recording Spectrophotometer. The infrared analysis was performed on a Nicolet FTIR Model 7000 Series Spectrometer. The mass spectrometers used were: CEC Model 110 High Resolution Mass Spectrometer, and the SCIEX, Ltd. Model TAGA-6000 MS/MS Atomspheric Pressure Ionization Source. The liquid chromatograph was a Waters High Performance Liquid Chromatograph equipped with Model 6000A solvent delivery system, Model 660 solvent programmer, Model U6K universal injector and Model 450 UV detector.

The materials were provided by the following: ground clove (McCormick Industrial Flavor Division); petroleum ether, ethyl acetate, ethyl ether, α,α'-dipyridyl (certified), purified ferric chloride anhydrous, linoleic acid, 2-propanol and acetic acid (Fisher Scientific Co.); ethanol, chloroform and methanol (Burdick & Jackson); chloroform and eugenol (Eastman); propyl gallate (NIPA Laboratories); gallic acid (Pfalz & Bauer); and polygram polyamide-6 UV254 for TLC, precoated plastic sheets 20 × 20 cm layer: 0.1 mm MN polyamide-TLC 6 UV254 with fluorescent indicator (Brinkman Instruments, Inc.).

EXPERIMENTATION

Extraction Procedure

A 150 g ground clove was packed into a glass chromatography column (500 mm × 35 mm). Two liters of petroleum ether were percolated through the column from a reservoir above. This was to remove any fat and much of the color pigments. This was followed by 2 liters of 80% ethanol to extract the phenolic compounds and sugars. The ethanol extract was concentrated on a rotary evaporator and extracted 3 times with ethyl acetate to remove the phenolic compounds and polar organics. Finally, the remaining ethanol solution was extracted 3 times with ethyl ether to remove the non-polar organic compounds. All reagents were tested for peroxides according to Vogel (23) before using. Each extract was dried on a rotary evaporator.

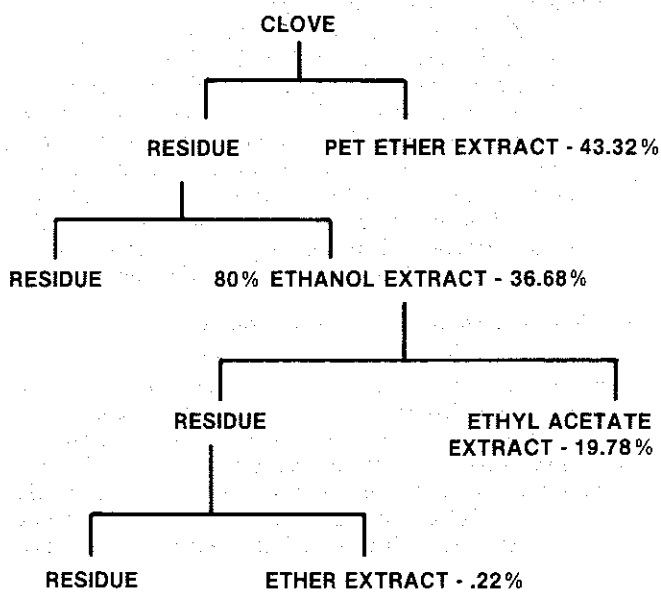

FIG. 1. Scheme for the extraction of antioxidants from clove.

Test for Antioxidant Activity

A 10 ml portion of each fraction was tested for antioxidant activity according to the method of Bishov (18). The ethyl acetate fraction showed the greatest activity and also gave a positive test when tested for the presence of phenols. This fraction was used for further fractionation. This ethyl acetate fraction accounts for 6.32% of the total sample.

Thin-Layer Chromatography

Using a precoated polyamide flexible sheet for TLC (24, 25), 5 microliters of a 10 mg/ml solution of the ethyl acetate fraction was spotted and developed in a solution of chloroform/methanol 1:1 v/v. One sheet was examined under ultraviolet light and was later sprayed with a 1% solution of α,α'-dipyridyl in ethanol followed by 1% ferric chloride in ethanol (26), a phenol detecting reagent. A spot one inch from the origin turned purple, indicating a phenolic compound was present. A second sheet was dotted with linoleic acid where spots had been seen in the UV on the first sheet and heated at 65 C overnight (27). This sheet had a white spot in the same position as the purple spot on the sprayed sheet, another indication of antioxidant activity. This spot did not fluoresce under long wave UV light, but quenched under the short wave UV light.

Preparative Thin-Layer Chromatography

Thirty plates were streaked with 50 μl of a 50 mg/ml sample of the ethyl acetate fraction and run in chloroform/methanol 1:1 v/v. The location of the antioxidant was marked under UV light and then removed from the plate and placed in a glass column connected to a reservoir. A 125 ml of 2-propanol was percolated through. The eluant was evaporated to dryness in a rotary evaporator and 6.6 mg of sample was recovered. This was dissolved in ethanol.

RESULTS AND DISCUSSION

An ultraviolet spectrum obtained showed one peak at 2725 Å. A literature search showed propyl gallate at 2750 Å and gallic acid at 2725 Å. The infrared spectra of the sample and gallic acid were comparable. The mass spectrum showed a molecular ion at 170.022 corresponding to $C_7H_6O_5$, which could be gallic acid, and a peak at 153 corresponding to a loss of an OH group.

Three TLC sheets were spotted with the sample, propyl gallate and gallic acid, and developed as before. The first plate was sprayed with α,α'-dipyridyl and ferric chloride. The gallic acid appeared in the same position as the extracted clove antioxidant. The propyl gallate moves further up the sheet. A second plate was sprayed with 10% linoleic acid in petroleum ether and heated at 65 C overnight. White spots appeared in the same position on the plate where the clove extract was spotted and where gallic acid was spotted. Propyl gallate gave a white spot at about twice the distance.

High Performance Liquid Chromatography

A Waters Liquid Chromatograph equipped with a 7.8 mm × 30 cm μBondapak C_{18} low polarity reverse phase column was used at slow flow rates. Optimum results were obtained by programming the liquid chromatograph for one hour at 1.5 ml/min using curve #3 and going from 100% Solvent A to 100% Solvent B. Solvent A was water and acetic acid 90:10 v/v and Solvent B was methanol, water and acetic acid 50:40:10 v/v/v. The ultraviolet detector was set at 2750 Å.

The eluted sample from the TLC plates was run as well as the original ethyl acetate fraction to see which peak was the antioxidant. Then propyl gallate, gallic acid and eugenol were run singly in order to compare their elution times with peaks on the original sample.

The eluted acetate fraction was spiked with 5 μl gallic acid, 20 mg/ml in methanol and 5 μl eugenol, 20 mg/ml in methanol and run. The tentatively identified peaks coincided with the standards, and identification was thus confirmed. By HPLC, the amount of gallic acid was determined to be 1.26 g per 100 g clove, and that of eugenol to be 3.03 g per 100 g clove.

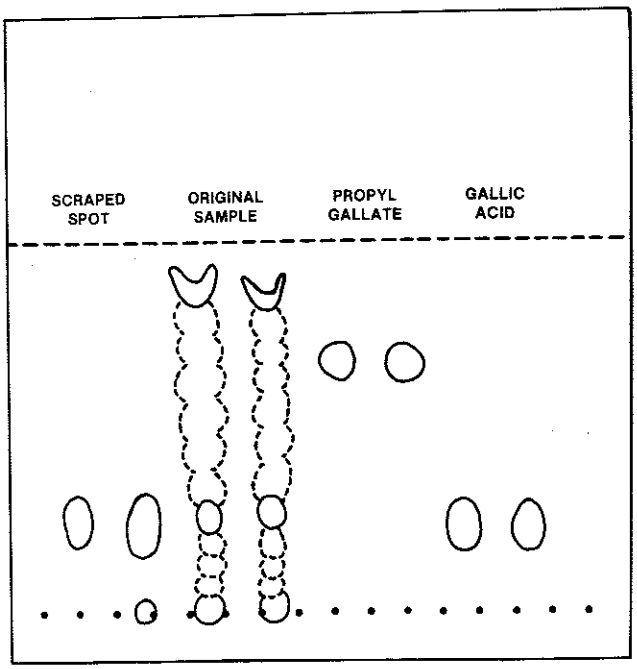

FIG. 2. Thin layer chromatogram of clove antioxidant fraction and standards.

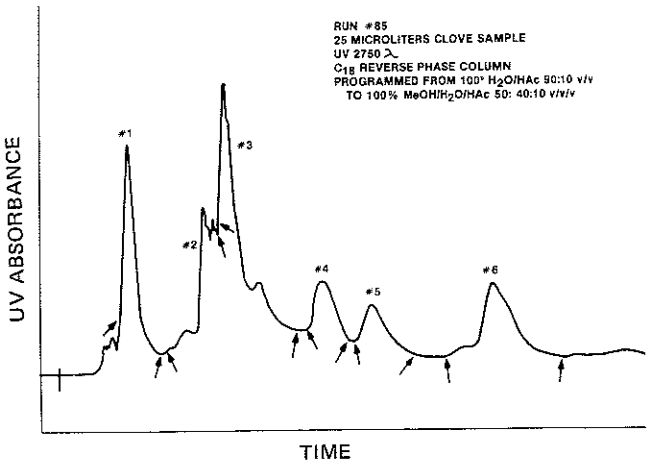

FIG. 3. Chromatogram of the ethyl acetate fraction of clove also showing the 6 areas collected.

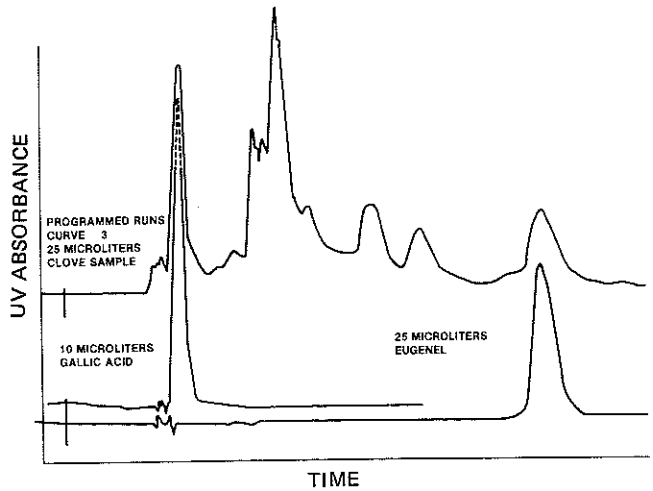

FIG. 4. Chromatograms showing the position of gallic acid and eugenol superimposed on the clove pattern.

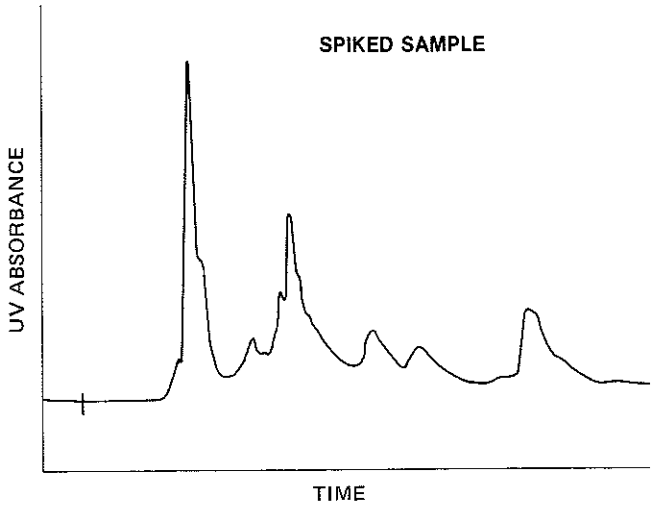

FIG. 5. Chromatogram showing the clove fraction that had been spiked with gallic acid and eugenol.

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